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ENTRAPMENT OF BACTERIOCIN 105B ONTO FABRIC WITH TITANIA

by
Robert Stote
and
Jennifer M. Rego

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U.S. Army Natick Soldier Research, Development and Engineering Center
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14. ABSTRACT
Current methods to fabricate multifunctional textiles to provide multiple facets of protection to the Warfighter are restrictive and not efficient. To address this challenge, previous work by the FORCE ProTex effort encapsulated a narrow-spectrum antimicrobial, the bacteriocin nisin, in a photocatalytic matrix, titanium dioxide (titania), on fabric to evaluate the creation of a multifunctional textile in unison. This report summarizes preliminary investigations to leverage this work by employing a different bacteriocin, an isolate from *Bacillus anthracis* termed 105b, to fabricate a multifunctional textile exhibiting an alternative range of antimicrobial activity from that of nisin, by titania encapsulation of 105b onto fabric. The results of these initial studies suggest that both pure preparations and semi-pure preparations of 105b are active when encapsulated in titania in solution. However, when the pure preparation of 105b is titania encapsulated on fabric, antimicrobial activity is not observed. It is hypothesized that the lack of activity of encapsulated pure 105b may be due to the removal of molecular stabilizers during the purification process that are responsible for maintaining the stability of the peptide, rendering it inactive. Further studies are necessary to confirm this mechanism. Additional work is also required to retain the activity of encapsulated pure 105b, ascertain the photocatalytic activity of the titania matrix, as well as elucidate the conditions for gradual release of the bacteriocin.

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Preface

This report documents work by the Biological Sciences and Technology Team (BSTT) of the Warfighter Directorate at the Natick Soldier Research, Development and Engineering Center (NSRDEC) during the period of September 2014 to March 2015. The effort detailed in this report focused on initial evaluations of fabricating a multifunctional textile by incorporating antimicrobial and photocatalytic functionalities into a single textile through the encapsulation of a narrow-spectrum antimicrobial, a bacteriocin, by precipitation with titania, a photocatalytic matrix, on a swatch of fabric. The resulting material is anticipated to exhibit both antimicrobial and photocatalytic properties. In this work, titania was precipitated in the presence of either a pure or semi-pure preparation of bacteriocin on a swatch of fabric. The resulting swatch of bacteriocin encapsulated titania composite was then evaluated for antimicrobial activity.

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ENTRAPMENT OF BACTERIOCIN 105B ONTO FABRIC WITH TITANIA

1 Introduction

The Biological Sciences and Technology Team (BSTT), Warfighter Directorate, of the Natick Soldier Research, Development and Engineering Center (NSRDEC) is investigating the application of narrow-spectrum antimicrobials in non-traditional, multifunctional textiles to protect the Warfighter from pathogenic bacteria. The Functional Oxides as Reactive Coatings for Enhanced Protection on Textiles (FORCE ProTex) effort has evaluated the encapsulation of the bacteriocin nisin, a narrow-spectrum antimicrobial peptide, in titantia, a photocatalytic matrix, on fabric to generate a multifunctional material that exhibits both antimicrobial and catalytic properties. To investigate the preparation of a multifunctional textile with a different antimicrobial spectrum, the findings of the FORCE ProTex efforts were leveraged and the feasibility of encapsulating an alternative bacteriocin, 105b, in the titania matrix on fabric to yield a multifunctional textile was evaluated. The objective of this technical report, was to detail the entrapment of bacteriocin 105b in titania coated on fabric and describe the resulting activity and stability of the 105b treated fabric. The work was performed from September 2014 to March 2015.

Traditionally, the military uses broad-spectrum antimicrobials in a wide array of applications to control pathogenic organisms. Broad-spectrum antimicrobials act not only on the target pathogenic organism but also kill beneficial bacteria that are necessary for inherent functions, such as wound healing. In addition, the application of broad-spectrum antimicrobials to textiles, polymer surfaces, wipes and ointments increases the development of multi-drug resistant strains of bacteria via the unintentional selection of strains which are resistant to the antimicrobial (Tattawasart *et al*. 1999, Thomas *et al*. 2000, McDonnell *et. al*. 1999, Silver 2003). The indiscriminate activity of the broad-spectrum antimicrobial eliminates benign bacteria that control the development of drug-resistant strains, permitting the resistant strains to thrive. Additional exposure to the broad-spectrum antimicrobial yields a bacterial pool comprised of predominantly resistant strains, rendering the broad-spectrum antimicrobial ineffective (Levy *et al*. 2004). Application of a new broad-spectrum antimicrobial to control the strains developing resistance to the original drug leads to further enrichment of the bacteria population for strains that are resistant to the new drug as well as the initially applied antimicrobial. Subsequently, "super bugs" that are multi-drug resistant develop, giving rise to a significant problem, especially in hospital settings.

As concern about the emergence of multi-drug resistant bacteria increases and the development of drugs to address the problem decreases, this and other research groups have been investigating new strategies to combat pathogenic bacteria and resistant strains. One promising strategy is to use narrow-spectrum antimicrobials, such as bacteriocins, as alternatives to broad-spectrum agents. Bacteriocins are narrow-spectrum bacterial toxins secreted by bacteria to kill other closely related bacteria that are competing for the same resources in their environment. Many

bacteriocins have very specific activity spectrums, as they only target one or two species. This narrow-range of selectivity offers a paradigm shift in applying antimicrobials, where instead of broadly killing all present organisms through the use of broad-spectrum antimicrobials, employing narrow-spectrum antimicrobials would facilitate specific pathogens to be targeted and beneficial bacteria to be unaffected and able to thrive (Abt *et al*. 2014). Generally considered a green alternative to currently used broad-spectrum antimicrobials, few bacteriocins have demonstrated adverse effects on eukaryote cells (Cotter *et. al*., Cox *et. al*., Galvez *et al*.). In previous work (Stote *et al. in press*), a bacteriocin with the sample name 105b was isolated and purified from *Bacillus subtilis*. This bacteriocin was found to be active against a surrogate strain for *Bacillus anthracis*, *Bacillus anthracis* Sterne. The activity spectrum for 105b was evaluated and discovered to be selective, primarily affecting *Bacillus* species. Because 105b demonstrated narrow-spectrum activity and a purification procedure has been established, this bacteriocin was selected for further studies in the use of a bacteriocin to prepare a multifunctional textile that exhibits both antimicrobial activity and an additional functionality.

Conventionally, fabrication of multifunctional protection for the Warfighter entails the development of separate components that each address an individual issue. A multifunctional platform is then achieved upon combination and integration of the individual components to form a single system that displays more than one property. While the current process for developing a multifunctional system does facilitate multiple threats to be addressed by one scheme, the resulting system can be clumsy and restricting. The individual elements to comprise a multifunctional system, each focused on eliminating a singular threat, may not be readily modular or may not function in the conditions necessary for another component upon integration. To overcome this challenge, the BSTT has been investigating novel, non-traditional textiles to advance the development of multifunctional materials for incorporation in protective garments. The goal is to prepare materials with multiple functionalities developed in unison to augment the protection of the Warfighter, while simultaneously diminishing the cost of production and the weight of the uniform. One strategy being examined to accomplish this aim is the encapsulation of biologics, such as bacteriocins, in a metal oxide matrix on fabric to simultaneously impart multiple functionalities to the textile.

The work detailed in this technical report conveys the results of preliminary studies on the encapsulation of bacteriocin 105b with the chosen encapsulation matrix of titanium dioxide, also known as titania. Titania exhibits photocatalytic properties, making it an excellent option for the foundation of a multifunctional bionanocomposite (Gaya *et al*. 2008). Previous studies have elucidated a facile method to precipitate titania under mild, biologically friendly conditions using biomimetic synthesis (Filocamo *et al*., 2011 and Luckarift *et al*., 2006). In these studies, an enzyme was successfully encapsulated in titania and demonstrated to retain its activity. In addition, work by the BSTT team (Filocamo) through the FORCE ProTex project has demonstrated the encapsulation of the bacteriocin nisin using titania precipitation. The work described in this report builds upon the findings of the FORCE ProTex effort by employing a similar methodology using bacteriocin 105b and evaluates the feasibility of encapsulating an alternative bacteriocin with a different narrow-spectrum range of antimicrobial activity.

2 Materials and Methods

2.1 Materials

Materials used in the course of this project include:

- Reputex™ from Lonza (Walkersville, MD)
- Nisin from ChiHonBio (Lisle, IL)
- NyCo from Annette LaFleur from the NSRDEC prototype shop
- Tryptone and mitomycin C from Amresco (Solon, OH)
- Sucrose from Alpha Aesar (Ward Hill, MA)
- Dipotassium phosphate and monopotassium phosphate from VWR (Randor, PA)
- Sodium citrate from JT Baker (Center Valley, PA)
- Tris, ammonium sulfate, magnesium sulfate, borax and titanium (IV) bis-(ammonium lactate)-dihydroxide (TBALD) from Sigma Aldrich (St. Louis, MO)
- 30,000 MWCO filter from EMD Millipore (Darmstadt, Germany)
- DEAE Sephadex A-25 column from GE Healthcare Bio-Sciences (Piscataway, NJ)
- *Bacillus anthracis* Sterne from Colorado Serum Company (Denver, CO)

2.2 Swatch Preparation

NyCo swatches were scoured in borax by boiling for 1 h. The swatches were then washed with water and air dried. Scoured swatches to be precipitated with titania were pre-treated by submerging the scoured swatches in 2.5% Reputex™ for 45 min. The swatches were removed from the Reputex™ and placed between six paper towels. The samples were "squeezed" between the paper towels to remove excess water. To bond the Reputex™ to the fabric, the samples were heated to 120 ^{o}C for 10 min.

2.3 Preparation of Bacteriocins

Two bacteriocins were used in this study: nisin and the 105b isolate.

Preparation of Nisin: The nisin stock solution was prepared by measuring out 0.125 g of 20% (w/v) nisin and adding it to 25 mL of 25 mM Tris buffer pH 7.5 to give a final concentration of 1 mg/mL of nisin. The nisin stock solution was vortexed for 5 min and spun down at 3000 rpm for 10 min. The supernatant was retained for future use.

Preparation of 105b: The 105b isolate was prepared for use as a semi-pure preparation and a pure preparation. To prepare the semi-pure preparation, a 10 mL nutrient culture was inoculated with the 105b isolate and grown for 4 h. This culture was used to inoculate a 1 L culture with 30 g tryptone, 5 g sucrose, 7 g dipotassium phosphate, 2 g monopotassium phosphate, 0.5 g sodium citrate, 0.1 g magnesium sulfate and 1 g ammonium sulfate. The culture was grown overnight at 37 ^{o}C. The cells were removed via centrifugation and the supernatant was retained. The supernatant was filtered through a 30,000 MWCO filter using tangential flow. The retentate was kept and used as the semi-pure preparation of 105b. To prepare the pure preparation of 105b, the retentate was further processed over a DEAE Sephadex A-25 column washed with 25 mM Tris buffer pH 8.5. As the 105b bacteriocin does not stick to the column, the pure peptide sample was collected in the flow through and subsequent washes of the column. To investigate the

effect of pH on the activity of bacteriocins, the pH of pure 105b was adjusted using hydrochloric acid to pH 4.5, 5.5 and 6.5 and then immediately evaluated for activity.

2.4 Titania Precipitation of Bacteriocins in Solution

Before precipitation with titania, the pH of bacteriocin solutions was adjusted to pH 6.5 with the addition of hydrochloric acid. To precipitate titania with bacteriocin in solution, 24.5 mL of bacteriocin solution was combined with 0.5 mL of titanium (IV) bis-(ammonium lactate)-dihydroxide (TBALD) pH 6.5. Precipitation occurred on a shaker at 250 rpm for 6 h. Titania precipitated bacteriocin solutions were then evaluated for activity without further purification.

2.5 Titania Precipitation of Bacteriocins to Encapsulate on Swatches

Reputex^{TM} treated swatches were divided among four Petri dishes with four swatches in each dish. To three Petri dishes 24.5 mL of a respective bacteriocin solution was added: nisin, pure 105b and semi-pure 105b. To the fourth Petri dish, 24.5 mL of Tris buffer was added to serve as a negative control. The Petri dishes were then placed on a shaker at 250 rpm until the swatches were wet thoroughly. To precipitate titania, 0.5 mL of TBALD was added to each Petri dish and titania was precipitated on the shaker for 6 h at 250 rpm. The supernatant was then poured off and 25 mL of DI water was added to each dish. The swatches were rinsed for 5 min and the supernatant removed. This step was repeated twice more so that the swatches were washed a total of three times.

2.6 Activity of Bacteriocins in Solution with and without Titania Encapsulation

A 10 mL culture of *Bacillus anthracis* Sterne was inoculated into nutrient broth and incubated at 37 ºC until an OD of 1 (~10^8 cfu/mL) was achieved. Soft agar for overlay experiments was prepared with 7% agar in nutrient broth. Soft agar overlays were prepared by adding 60 µL of *Bacillus anthracis* Sterne with 1.35 µL of a 100 mg/ml stock mitomycin C solution to a 7 mL aliquot of soft agar. Bacteriocins in solution with and without the precipitation of titania were tested for activity by dropping 6 µL of bacteriocin solution onto each plate. The plates were incubated overnight at 37 °C. Positive activity was determined by the presence of a zone of clearing.

2.7 Activity of Titania Encapsulated Bacteriocin Swatches

Soft agar overlays were prepared as described previously in 2.6. One set of swatches was immediately put onto the soft agar overlay after titania precipitation (0 h) and incubated overnight at 37 ºC. Another set of swatches was incubated at 4 ºC for 24 h and then placed onto a soft agar overlay and incubated overnight at 37 °C. Positive activity was determined by the presence of a zone of clearing around the swatch.

3 Results and Discussion

3.1 Activity of Bacteriocins in Solution

The antimicrobial activity of solutions of nisin, semi-pure 105b and pure 105b was confirmed using the soft agar overlay method. Figure 1A illustrates the zones of clearing observed for each bacteriocin against *Bacillus anthracis* Sterne. As expected, all the bacteriocin solutions tested exhibited activity, with nisin and pure 105b exhibiting the greatest zones of clearing. Though the semi-pure 105b did exhibit a zone of clearing, it was visibly smaller than the zones observed for nisin and pure 105b, suggesting that the semi-pure 105b exhibits less activity than pure 105b and nisin. This finding is expected as the semi-pure sample of 105b was subject to less purification. This resulted in a smaller concentration of 105b in the protein content of the semi-pure 105b sample in comparison to the pure preparation of the 105b sample whose protein content is mostly the 105b peptide.

To determine the effect of pH on the activity of 105b, the pH of pure 105b was adjusted to 4.5, 5.5 and 6.5 and then immediately evaluated for activity. As seen in Figure 1A, the pure preparation of 105b at each pH tested exhibited activity as inferred from the observation of zones of clearing. This observation indicates that the activity of pure 105b is retained over the range of pHs tested. Additionally, the solution of 105b at various pHs was stored at 4 °C for several weeks before again being evaluated for activity. Activity was still observed even under these conditions (results not shown) indicating that the pure 105 remains stable and active when stored at the pH range tested.

The activity of bacteriocins encapsulated in titania in solution was evaluated by the soft agar overlay method against *Bacillus anthracis* Sterne. Figure 1B depicts the results.

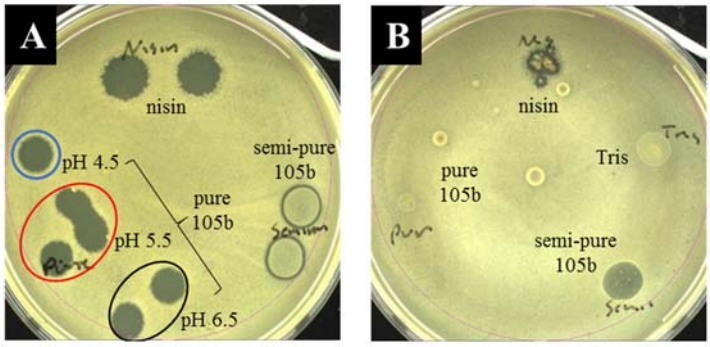

Figure 1. Activity of bacteriocins in solution. A) Activity of bacteriocins in solution without encapsulation in titania. Samples tested were nisin, semi-pure 105b and pure 105b at pH 6.5 (black circle), 5.5 (red circle) and 4.5 (blue circle). The colored circles have been added to assist with visualization. B) Activity of titania encapsulated bacteriocins in solution after titania precipitation. Samples tested were nisin, Tris buffer (negative control), semi-pure 105b and pure 105b.

As expected, titania precipitated in the absence of a bacteriocin did not yield a zone of clearing, indicating the titania matrix is not antimicrobially active. This finding confirms that titania is a suitable encapsulation medium for the bacteriocins. The precipitated titania with nisin exhibited

a zone of clearing, indicating that the activity of nisin is retained when entrapped. This finding is consistent with results of the FORCE ProTex effort (Sherman *et al.* in process). Although semi-pure 105b appears to exhibit less activity than pure 105b in solution (Figure 1A), when precipitated with titania, the encapsulated semi-pure 105b retained activity while pure 105b did not. The difference in activity observed for the encapsulated 105b samples may be due to the presence of contaminating elements and/or additional proteins in the semi-pure preparation of 105b. These elements may act as molecular stabilizers which protect the peptide from degradation during the precipitation process, preserving the activity of the peptide. Additionally, the lack of activity observed for the encapsulated pure 105b material may be due to the absence of these stabilizers, which may have been stripped away during the purification process, promoting the inactivity of the pure peptide. Also of particular note, the semi-pure 105b appears to exhibit greater activity when encapsulated in titania than when not encapsulated. Several hypotheses may explain this observation. The titania precipitate may disrupt potential aggregation of the peptide in solution, resulting in more uninhibited, active peptide to be encapsulated. Also, the precipitation of titania may draw the bacteriocins from solution to encapsulate a high concentration of bacteriocin, resulting in the observation of greater activity. Additional studies are required in order to investigate and ascertain the mechanism responsible for the increased activity of semi-pure 105b when encapsulated in precipitated titania.

3.2 Activity of Titania Encapsulated Bacteriocin Swatches

To investigate the development of a multifunctional textile, NyCo swatches with titania encapsulated bacteriocins were tested for activity using soft agar overlays against *Bacillus anthracis* Sterne. The presence of activity was noted by a zone of clearing observed around the swatch. Since Reputex™, the precipitation inducing agent, exhibits antimicrobial activity, swatches only treated with Reputex™ were first evaluated to determine the extent titania precipitation could cover the surface and eclipse the antimicrobial activity of Reputex™. Figure 2 shows the results of this first study. All the swatches evaluated were tested for activity immediately following precipitation (Figure 2A, 0 h) and after incubation for 24 h at 4 °C (Figure 2B, 24 h). Untreated swatches (UT) of NyCo did not exhibit any activity verifying that the NyCo fabric is not antimicrobial. Swatches treated with Reputex™ without titania precipitation (R) exhibited a zone of clearing around the perimeter of the swatch signifying activity, while swatches treated with Reputex™ in the presence of titania precipitation (R+Ti) did not. This confirms that the precipitation of titania successfully masked the antimicrobial activity of the Reputex™ on the treated swatches at both time points tested.

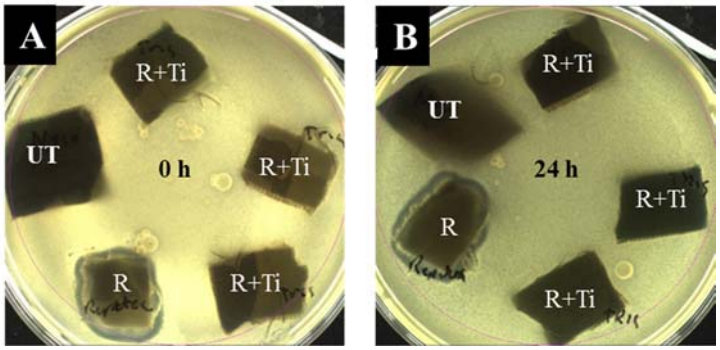

Figure 2. Activity of control fabric with and without titania. Samples tested were Reputex™ treated swatches precipitated with titania in only Tris buffer (R+Ti), a Reputex™ treated swatch without titania precipitation (R) and an untreated swatch (UT). A) Activity of control swatches treated with titania encapsulated bacteriocins right after treatment (0 h). B) After the treated swatches were incubated for 24 h at 4 °C.

The activity of bacteriocins entrapped by titania precipitation on Reputex™ treated NyCo swatches was evaluated using the soft agar overlay method against *Bacillus anthracis* Sterne. As seen in Figure 3, swatches with nisin encapsulated by titania exhibited a zone of clearing around the swatch, indicating that the bacteriocin remains active following precipitation with titania. The nisin encapsulated swatches retain their activity even after incubation at 4 °C for 24 h. These findings are in agreement with previous studies evaluating activity of swatches coated with titania encapsulated nisin completed by the FORCE ProTex effort. The activity of semi-pure 105b and pure 105b encapsulated by titania precipitation on Reputex™ treated swatches was also investigated. Figure 4 shows the activity of swatches with titania encapsulated semi-pure 105b, while Figure 5 shows the activity of swatches with titania encapsulated pure 105b. As seen in Figure 4, after immediate testing and after storage at 4 °C for 24 h, one of the three swatches with titania encapsulated semi-pure 105b demonstrated activity as evidenced by the zone of clearing around the swatch highlighted by the added arrows in the figure. No activity was observed for swatches encapsulated with pure 105b as seen in Figure 5. These results are in agreement with the observations for titania encapsulated semi-pure and pure 105b in solution (Figure 2B), where only the semi-pure 105b encapsulated in titania demonstrated activity. As with the precipitation of titania in the presence of the bacteriocins in solution, the difference in activity observed for the semi-pure 105b and pure 105b preparations is hypothesized to be due to the difference in the preparation conditions for the two samples. The remaining impurities in the semi-pure preparation of 105b may act as a stabilizer that keeps the peptide active. As these impurities are removed during further purification to obtain pure 105b preparation, the peptide may become unstable, resulting in a loss of activity. This has been seen with other peptides and proteins. Further testing is necessary to confirm this hypothesis and investigate alternative conditions to promote the antimicrobial activity of titania encapsulated purified 105b on swatches.

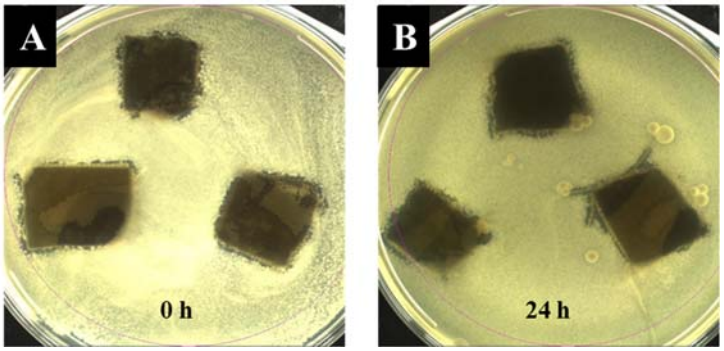

Figure 3. Activity of nisin entrapped in titania on fabric. A) Activity of swatches treated with titania encapsulated nisin right after treatment (0 h). B) After the treated swatches were incubated for 24 h at 4 °C.

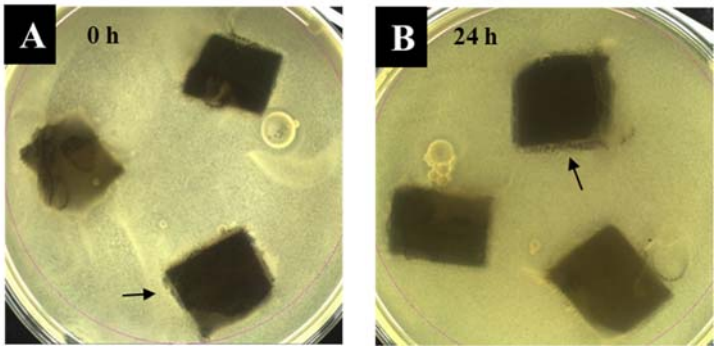

Figure 4. Activity of semi-pure 105b entrapped in titania on fabric. A) Activity of swatches treated with titania encapsulated semi-pure 105b right after treatment (0 h). B) After the treated swatches were incubated for 24 h at 4 °C. Arrows have been added to point out the observed zones of clearing around the swatches.

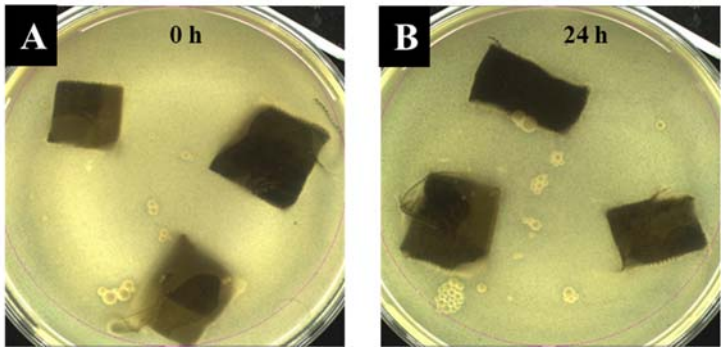

Figure 5. Activity of pure 105b entrapped in titania on fabric. A) Activity of swatches treated with titania encapsulated pure 105b right after treatment (0 h). B) After the treated swatches were incubated for 24 h at 4 °C.

4 Conclusions

Previous work developing a universal purification protocol for quickly characterizing bacteriocins was leveraged as the foundation for this work, similar to purification methods employed by industry (Immucell, Portland, ME). This purification method employed tangential flow filtration to rapidly yield a crude bacteriocin sample with enough purity to evaluate activity. This process is advantageous to purification procedures developed in the laboratory, which predominantly employ precipitation by ammonium sulfate (Pingitore *et al*. 2007) and do not readily translate to large-scale industrial applications due to high cost and labor. As detailed in this report, building upon the universal purification protocol, a purification protocol specific to bacteriocin 105b was achieved. Tangential flow filtration in conjunction with anion exchange column chromatography was employed. Initial characterization of the ensuing sample of antimicrobial peptide suggests a high yield of bacteriocin 105b was acquired. Further evaluation is necessary to confirm the purity and establish the quantity produced of bacteriocin 105b. The heterogeneous nature of bacteriocins as well as the numerous and varying protocols employed to purify bacteriocins makes it difficult to compare the results of this purification protocol to others (Carolissen-Mackay *et al*. 1997). Furthermore, bacteriocin 105b is an environmental isolate whose identity is currently unknown.

In future studies regarding bacteriocin 105b, the antimicrobial peptide should be sequenced to ascertain if the antimicrobial peptide is novel or has been previously characterized in literature. The use of bacteriocin 105b is envisioned in a textile that demonstrates antimicrobial activity to protect the Warfighter from pathogenic bacterial threats. To accomplish this goal, the stability of bacteriocin 105b in a textile must be evaluated and supplemented as necessary to ensure the antimicrobial peptide remains active. Long-term stability and activity of bacteriocin 105b should be established to determine the effects of potential storage conditions of the antimicrobial textile in an employable environment. Additionally, the activity of bateriocin 105b against commensal flora, as well as the identity of any remaining impurities in the extract of bacteriocin 105b, must be evaluated to ensure the safety of the Warfighter. The development of a purification protocol specific to bacteriocin 105b to yield a highly pure antimicrobial peptide with narrow-spectrum activity is a significant step towards advancing a novel construct to provide antimicrobial protection to the Warfighter.

This document reports research undertaken at the U.S. Army Natick Soldier Research, Development and Engineering Center, Natick, MA, and has been assigned No. NATICK/TR- 17/008 in a series of reports approved for publication.

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